E. 1 Isolation of sMMO genes form *M. capsulatus*

Standard Protocols:

QuikChange II Site-Directed Mutagenesis Kit

In order to isolate all subunit encoding genes of the sMMO from *M. capsulatus* colony PCR with specifically designed primers is performed. Additionally we attempt to isolated some chaperones that might be important for the correct folding of the sMMO from *M. capsulatus*.

14.04.2014

- Dilution of Primer 1-40 as well as 1995, 1996, 2036, 2037 1:10-dilution (20 µL stock solution + 180 µL H_2O) → except for primer 1996 only 10 µL + 90 µL
- Colony PCR of 11 genes (groEL, groEL2, groEL3, groES, groES2, mmoB, mmoC, mmoD, mmoX, mmoY, mmoZ) with each 50 µL per PCR reaction
- Gel electrophoresis of all 11 PCR-reations (130 V)
    - 0,8% agar -> reactions 1, 2, 3, 7, 9 and 10 [Gel 1]
    - 1,5% agar -> reactions 4, 5, 6, 8 and 11 [Gel 2]

reaction	Gen	gen-length	Primer pair	Success
1	groEL	1659 bp	41/42	No
2	groEL2	1733 bp	43/44	No
3	groEL3	1659 bp	45/46	No
4	groES	239 bp	47/48	Yes
5	groES2	345 bp	49/50	No
6	mmoB	480 bp	51/52	Yes
7	mmoC	1101 bp	53/54	Yes
8	mmoD	366 bp	55/56	Yes
9	mmoX	1638 bp	57/58	Yes
10	mmoY	1224 bp	59/60	Yes
11	mmoZ	567 bp	61/62	Yes

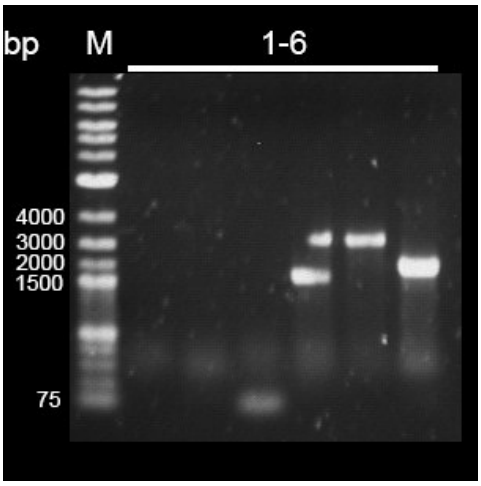


Fig. 1.1 *Colony-PCR – **1**: groEL (Exp. Length 1659 bp); **2**: groEL2 (Exp. Length 1733 bp); **3**: groEL3 (Exp. Length 1659 bp); **4**: mmoC (Exp. Length 1101 bp); **5**: mmoX (Exp. Length 1638 bp); **6**: mmoY (Exp. Length 1224 bp)*

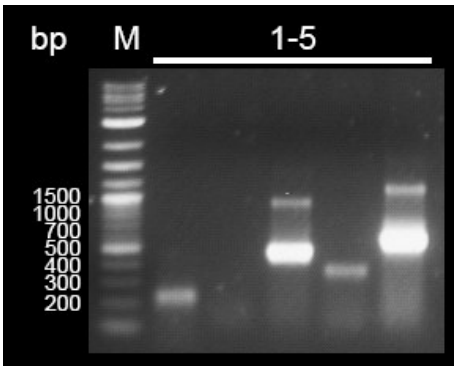


Fig. 1.2 *Colony-PCR – **1**: groES (Exp. Length 239 bp); **2**: groES2 (Exp. Length 345 bp); **3**: mmoB (Exp. Length 480 bp); **4**: mmoD (Exp. Length 366 bp); **5**: mmoZ (Exp. Length 567 bp)*

Purification of gel of successful colony PCR reactions of 4 and 6-11 in 1% agar gel

- pattern (each 5 µL): marker-4-4-X-6-6-X-…-11 (only once 15 µL)-marker
- gel electrophoresis at 130 V
- cutting out of DNA fragments an purification with Wizard SV Gel Kit (Promega)
- elution in each 30 µL H_2O

Afterwards all genes are cloned into the standard iGEM expression vector pSB1C3.

24.04.2014

Because some sMMO genes contain restriction sites that are prohibited in RFC 10 assembly standard, we perform site directed mutagenesis to remove these restriction sites using QuikChange II Site-Directed Mutagenesis Kit.

To be mutated:

1. 1x groES iGEM 2014-4.3.4.(1/2)

2. 1x mmoC iGEM 2014-4.3.2.(1/9)
3. 2x mmoX iGEM 2014-4.3.9.(9/13)
4. 1x mmoY iGEM 2014-4.3.10.(1/5)

Mutations are being conferred by Quick Change Mutagenesis.

Primer:

1. 71/72
2. 73/74
3. 75/76
4. 79/80

Afterwards Dpn I digestion for 2 h and heat-shock with 10 µL.

Plating of the whole samples.

- No colonies could be observed on respective plates. It was recognized that the volume of sample was too small. Probably addition of 50 µL was forgotten and therefore the DNA-polymerase could not work, probably due to too high Mg^{2+} concentration. The whole experiment was therefore repeated.

28.04.2014

The following table shows the used quantities used for the site directed mutagenesis in µL.

	groES		**mmoC**		**mmoX**		**mmoY**	
Reaction buffer Pfu 10x	5	5	5	5	5	5	5	5
dsDNA template 50 ng	0,352	0,254	0,980	0,543	0,318	0,223	0,303	0,277
Primer 1	1,25	1,25	1,25	1,25	1,25	1,25	1,25	1,25
Primer 2	1,25	1,25	1,25	1,25	1,25	1,25	1,25	1,25
dNTP mix	1	1	1	1	1	1	1	1
ddH_2O	40,148	40,246	39,520	39,957	40,182	40,277	40,197	40,227
Pfu	1	1	1	1	1	1	1	1

Polymerase								
∑	50	50	50	50	50	50	50	50
Used Primers	71/72		73/74		75/76		79/80	

30.04.2014

Colony PCR of mutated (one Pst1 restriction site removed):

- iGEM 4.3.4.1/2 groES
- iGEM 4.3.9.13 mmoX
- iGEM 4.3.10.1/5 mmoY

Used Primers: P83/84

Master-Mix and PCR-Program:

	Comment	Amount per Sample [µL]	Amount per 65 Samples [µL]
Template	Colony	0,25	16,25
5x GoTaq-Buffer	No Mg2+	2	130
10 mM dNTPs		0,2	13
Primer 83	10 pmol per µL	0,5	32,5
Primer 84	10 pmol per µL	0,5	32,5
GoTaq Polymerase	5 U per µL (contains Loading Buffer)	0,05	3,25
MgCl		0,8	52
dH2O		5,7	370,5
Sum		10	650

	Temperature	**Time [min]**
1	95 °C	2:00
2	94 °C	0:15

3	56 °C	0:20
4	72 °C	1:10
5	Goto step 2 24x	
6	72 °C	3:00
7	16 °C	For ever

Restriction of Pst1-sites (per sample):

- 10 µl PCR-sample
- 2 µl Cutsmart-Buffer
- 0,2 µl Pst1

} x60 as master mix

- 7,8 µl h2o

Gelelectrophoresis

- 4.3.4.1/2 in 2,5% agarose-gel
- The rest in 1% agarose-ge

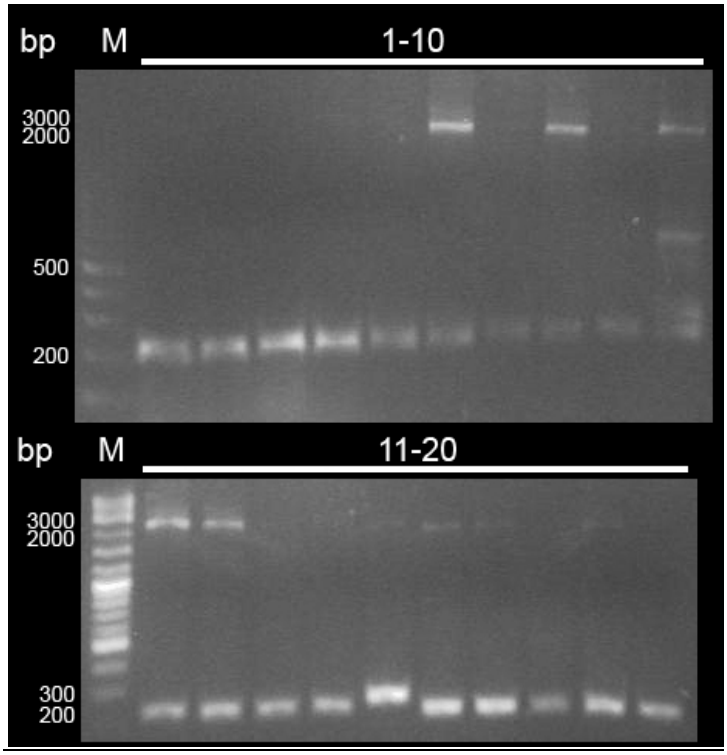


Fig. 1.3 *Test-restriction with Pst1 (in 2,5% agarose gel) –* ***1-10****: groES Clone 1;* ***11-20****: groES Clone 2*

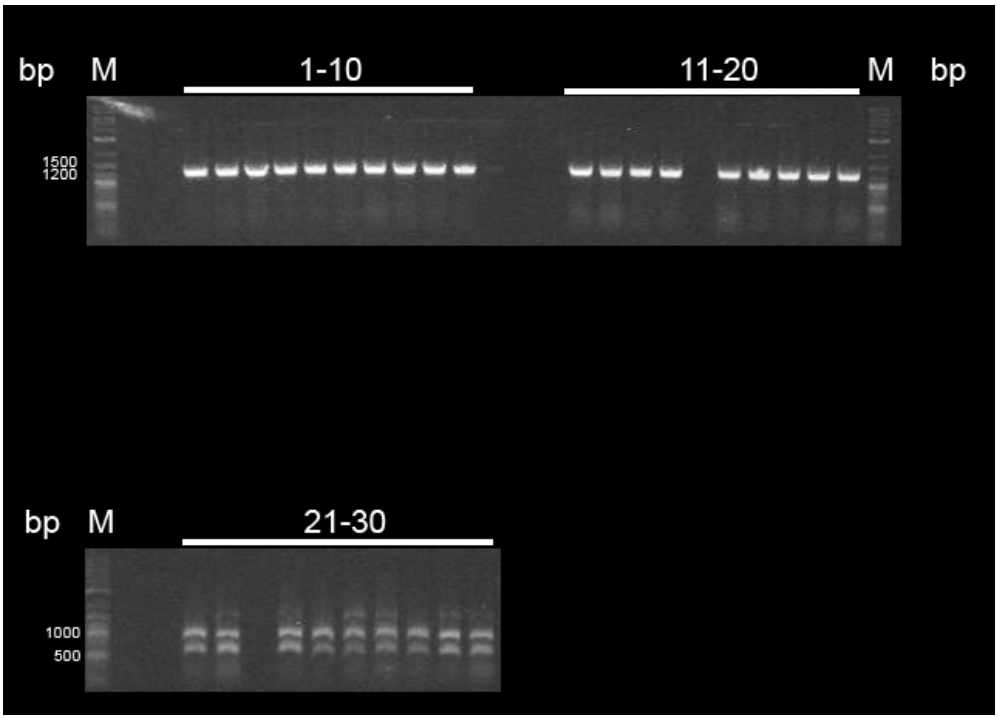


Fig. 1.4 *Test-restriction with Pst1 –* ***1-20****: mmoX;* ***21-30****: mmoY (both in 1% agarose gel)*

04.05.2014

Overnight culture of:

- 4.3.4.1-1/2 (groES)
- 4.3.4.2-3/4 (groES)
- 4.3.9.13-1/2 (mmoX)
- 4.3.10.1-1/2 (mmoY)
- 4.3.10.5-1/2 (mmoY)

05.05.2014

Miniprep of all experiments:

- Changes: After lysis and neutralization centrifuge at 13.000 x g for 10 min (instead of 11.000 x g for 5 min)

Sample	**Plasmid-Concentration [ng/µl]**	**Number in freezer**
4.3.4.1-1	351.27	39
4.3.4.1-2	190.11	40
4.3.4.2-3	101.56	41

4.3.4.2-4	176.54	42
4.3.9.13-1	213.25	43
4.3.9.13-2	388.15	44
4.3.10.1-1	333.89	45
4.3.10.1-2	185.95	46
4.3.10.5-1	322.30	47
4.3.10.5-2	578.41	48

Glycerinstock of:

Sample	**Number in freezer**
4.3.4.1-1	38
4.3.4.1-2	39
4.3.4.2-3	40
4.3.4.2-4	41
4.3.9.13-1	42
4.3.9.13-2	43
4.3.10.1-1	44
4.3.10.1-2	45
4.3.10.5-1	46
4.3.10.5-2	47

Mutation-PCR of:

- 4.3.9.13-1 (mmoX), second mutation with primer 77/78
- 4.3.9.13-2 (mmoX), second mutation with primer 77/78
- 4.3.7.10 (mmoC), first mutation with primer 73/74

PCR-Mix:

	4.3.9.13-1 (mmoX)	4.3.9.13-2 (mmoX)	4.3.7.10 (mmoC)

Reaction buffer Pfu 10x	5 µl	5 µl	5 µl
dsDNA Template – 5ng	2,3 µl	1,3 µl	5,6 µl
Primer 1	1,25 µl	1,25 µl	1,25 µl
Primer 2	1,25 µl	1,25 µl	1,25 µl
dNTP mix	1 µl	1 µl	1 µl
ddH2O	38,2 µl	39,2 µl	34,9 µl
Pfu Polymerase	1 µl	1 µl	1 µl
Sum	50 µl	50 µl	50 µl

Dpn1-digestion:

To each PCR-sample 1 µl of Dpn1 restriction enzyme, mix and incubate at 37 °C

Transformation:

Heat shock transformation of the 3 dpn1-digested samples. Each sample is completely distributed on its own plate.

06.05.2014

Colony PCR

Mutated constructs from transformation the day before:

- iGEM 4.3.9.13-1 (mmoX)
- iGEM 4.3.9.13-2 (mmoX)

} 10 PCR-samples with 10 different colonies

- iGEM 4.3.7.10 (mmoC)

Used Primers: P86/87

Master-Mix and PCR-Program:

	Comment	Amount per Sample [µL]	Amount per 35 Samples [µL]

Template	Colony	0,25	8,75
5x GoTaq-Buffer	No Mg2+	2	70
10 mM dNTPs		0,2	7
Primer 83	10 pmol per µL	0,5	17,5
Primer 84	10 pmol per µL	0,5	17,5
GoTaq Polymerase	5 U per µL (contains Loading Buffer)	0,05	1,75
MgCl		0,8	28
dH2O		5,7	199,5
Sum		10	350

	Temperature	**Time [min]**
1	95 °C	2:00
2	94 °C	0:15
3	56 °C	0:20
4	72 °C	1:10
5	Goto step 2 24x	
6	72 °C	3:00
7	16 °C	For ever

<u>Restriction of Pst1-sites (per sample):</u>

- 10 µl PCR-sample
- 2 µl Cutsmart-Buffer
- 0,2 µl Pst1

} x30 as master mix

- 7,8 µl h2o

1h , 37 °C

<u>Gelelectrophoresis of colony PCR samples</u>

In 1% gel

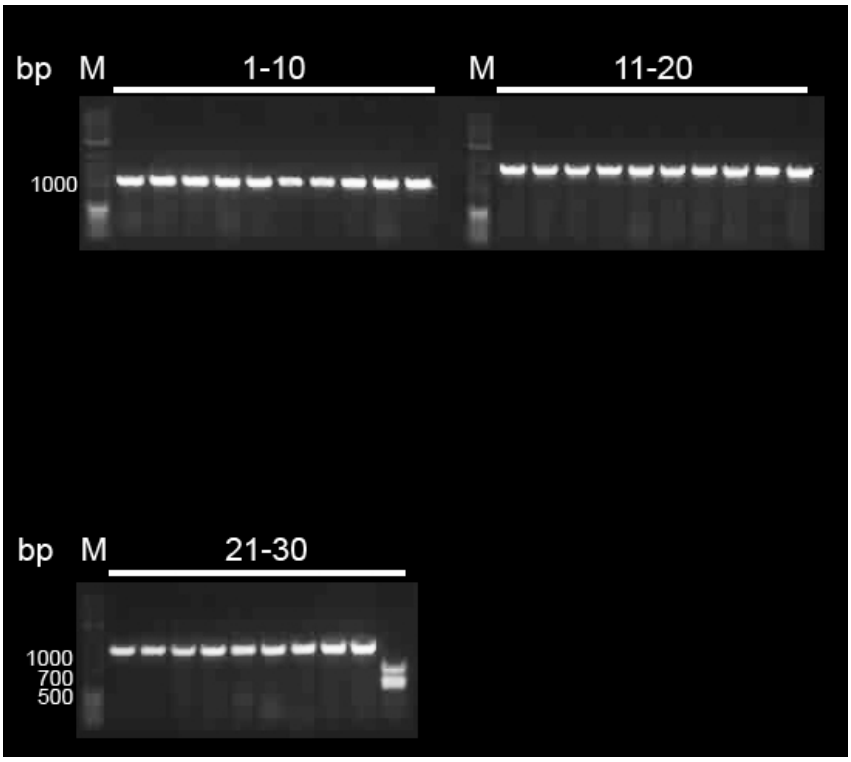
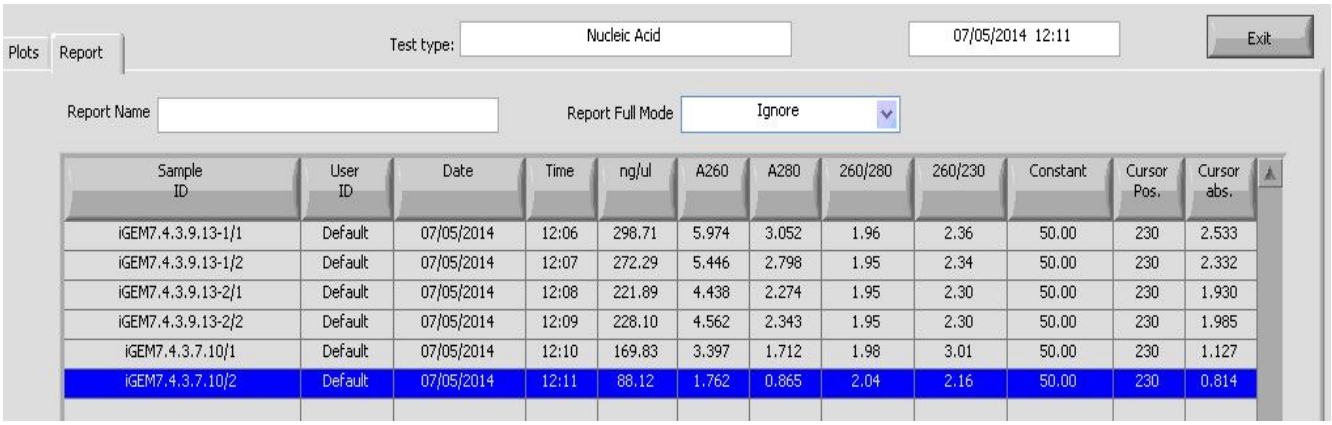


Fig. 1.5 *Test-restriction of Colony-PCR with Pst1 (in 1% agarose gel) –* ***1-10****: mmoC;* ***11-20****: mmoX Clone 1;* ***21-30****: mmoX Clone 2*

<u>Overnight culture 37°C</u>

- With picked colonies of the first two successful samples (from each of the three constructs) of the PCR made earlier

<u>Plasmid-Prep of overnight cultures</u>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Report Name Report Full Mode Ignore

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iGEM7.4.3.9.13-1/1	Default	07/05/2014	12:06	298.71	5.974	3.052	1.96	2.36	50.00	230	2.533
iGEM7.4.3.9.13-1/2	Default	07/05/2014	12:07	272.29	5.446	2.798	1.95	2.34	50.00	230	2.332
iGEM7.4.3.9.13-2/1	Default	07/05/2014	12:08	221.89	4.438	2.274	1.95	2.30	50.00	230	1.930
iGEM7.4.3.9.13-2/2	Default	07/05/2014	12:09	228.10	4.562	2.343	1.95	2.30	50.00	230	1.985
iGEM7.4.3.7.10/1	Default	07/05/2014	12:10	169.83	3.397	1.712	1.98	3.01	50.00	230	1.127
iGEM7.4.3.7.10/2	Default	07/05/2014	12:11	88.12	1.762	0.865	2.04	2.16	50.00	230	0.814

Finally, all sMMO genes are inserted into the pSB1C3 expression vector and ready to be used by the entire iGEM community.